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IN VITRO EVALUATION OF ANTIOXIDANT POTENTIAL AND *IN VIVO* STUDIES FOR PRECLINICAL TOXICITY WITH HEPATOPROTECTIVE ACTIVITY OF SAFOOF-E-AKSEER-E-JIGAR

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ABSTRACT

To Evaluate the *in vitro* and *in vivo* antioxidant potential and hepatoprotective activity of Safoof-e-Akseer-e-Jigar (SAJ). The antioxidant study of SAJ was carried out i*n vitro* by using 1,1-diphenyl-2-picryldrazyl (DPPH) free radical scavenging method. Acute oral toxicity was evaluated in Swiss albino mice to determine the maximum Tolerated Dose (MTD). Hepatoprotective activity was evaluated by paracetamol induced liver injured animal model in Wistar rats. Acute oral toxicity study revealed that SAJ is a safe compound as none of the animals died during the observation period and there were no sign and symptoms of toxicity related to it and its MTD>2600mg/kg in mice indicating its high safety profile. SAJ at a dose of 180mg/kg, was found to have significant ($p<0.05$) hepatoprotective activity by the inhibition of elevated biomarkers of paracetamol induced liver damage viz., SGOT, SGPT, ALP, and SB apart from maintaining normal haemoglobin levels and was found to be comparable with that of Liv 52 which was confirmed by the histopathological examination of liver of rats and also revealed on pathological changes even after administration of SAJ for 7 days. The present studies

demonstrate the potential Anti-oxidant and Hepatoprotective properties of Safoof-e-Akseer-e-Jigar.

KEYWORDS: Safoof-e-Akseer-e-Jigar (SAJ), Antioxidant, Hepatoprotective.

INTRODUCTION

Liver plays a major role in metabolism and excretion and involved in carbohydrate, protein, fat metabolism, synthesis of several enzymes responsible for various biochemical pathways, storage of vitamins, detoxification of endogenous and exogenous substances, bile secretion etc. It also helps in maintaining and regulating homeostasis of the body. If liver turnout to be diseased or injured either viral or non-viral means, it leads to inflammation and severe complications involving different systems of the body which will be life threatening. The liver diseases are considered as one of the major cause of fatality in the world. It may be acute or chronic disease. Acute viral hepatitis include viruses mostly HBV-DNA and rarely HAV. Exposure of liver to toxins, chemicals, drugs and alcohol will lead to non-viral acute hepatitis.[1] The commonly used antipyretic and analgesic drug is acetaminophen (Paracetamol), because of its easy availability at medical stores without a prescription. The drug is safe when taken at therapeutic doses. However its overdose may cause toxicity resulting in necrosis of the hepatic cells and it may be fatal. Early symptoms are mild and non specific such as nausea, vomiting and liver tenderness. Initially the patient is asymptomatic or has mild gastrointestinal upset. The centro-lobular hepatic necrosis occurs after 12 to 18 hours of ingestion of the drug, raised levels of bilirubin after 2 days and finally increases aminotransferase level and hypo prothrombinemia leading to liver injury.[2,3] The drug induces oxidative stress leading to generation of free radicals in the form of reactive oxygen species (ROS) in the body. Several studies have reported that accumulation of ROS in biological system causes oxidative damage to tissue that affects cellular integrity and functions resulting in diseases like aging, cardiovascular diseases, liver disorders, cancer and stroke etc.[4] The treatment of liver disorders by modern medicinal drugs would alleviate the symptoms to a lesser extent. Therefore there is a need to strengthen and protect the liver from further damage. In the traditional system of medicine, many plants and polyherbal formulations were reported to be hepatoprotective and have potential to treat liver diseases. Unani physicians have put forth the organ Jigar as Azu-Raees (vital organ). In Unani medicine single or combination of herbs are used to treat liver disorders. In the present study the liver disorder was treated by using Unani herbo-mineral formulation Safoof-e-Akseer-e-

Jigar (SAJ)[5,6], contain standardized plant which is known to be hepatoprotective, hepatostimulant, hepatocorrective.

MATERIALS AND METHODS

SAJ is a compound drug consists of combination of the following in the ratio 1/3rd of each (Table 1) The compound SAJ is taken orally by the patients in the form of powder.

Table 1: Formulation Composition details (clinical)

Drug code	Ingredients	Origin	Ratio	Human dose
Safoof-e-Akseer-e-Jigar (SAJ)	Revand (*Rheum emodi*)	Root	1/3rd	1000 mg/bid (or) 2000 mg/daily[5]
	Naushader (Ammonium chloride)	Mineral	1/3rd	
	Shora Qalmi (potassium Nitrate)	Mineral	1/3rd	

The present study was carried out in animal models. The study was approved by Institutional animal ethical committee (IAEC) Lr. No.3/IAEC/P.G Advia /2009 of Govt Nizamia Tibbi College, Charminar, Hyderabad, Telangana. The experimental work was performed in accordance with the guidance of CPCSEA.

In vitro evaluation of anti oxidant potential of SAJ[7,8,9,10,11,12,13]

A simplest method followed to determine the antioxidant activity of polyherbal compound utilizes the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. The odd electron in the DPPH free radical gives a strong absorption maximum at 517nm and is purple in color. The color turns from purple to yellow as the molar absorbtivity of the DPPH radical reduces from 9660 to 1640 at 517nm. The DPPH radical get reduced to DPPH-H when it comes in contact with hydrogen of a free radical scavenging antioxidant.

The DPPH free radical scavenging activity was measured using the following method.

Briefly, all the stock test extracts were dissolved in DMSO at concentration of 10mg/ml (150 µg/ml in final reaction mixture) and are serially diluted in DMSO by two fold including the standard (Ascorbic acid) at a concentration of 1mg/ml. DPPH is prepared freshly in absolute alcohol at a concentration of 4.9mg/25ml. The reaction mixture consisted of 125µl of DPPH,100µl of freshly prepared 0.5 mm Tris buffer (pH 7.2) and 25µl test extracts or standard is added to 96 well plates. The plate is incubated at room temperature for 10min then absorbance is measured at 517 mm by a UV-visible Spectrophotometer.[7,8,9]

***In vivo* acute oral toxicity and hepatoprotective studies of SAJ**

Swiss Albino mice of both sexes weighing 18-20gms and Wistar rats of both sexes weighing 160-180gms were procured from a private laboratory animal suppliers, Mahaveera enterprises, Hyderabad Govt.Reg.No.146/1999/CPCSEA. The animals were housed in conditioned environment with controlled temperature of 23 ± 2 ^{o} C and humidity of 50 ± 5)% with a 12–12 h light–dark cycle.[14] The animal had access to food and water ad libitum before acute hepatotoxicity.[15] The experiment was carried out to study the acute toxicity and hepatoprotectivity of the compound, SAJ according to the method by Organization for Economic Cooperation and development (OECD).[16,17] The animals were administered the compound at a fixed dose orally for 14 days and 7 days respectively. 10 Swiss Albino mice (5 males and 5 females) were used. Animals were fasted over night and exposed to the test compound (once 10 x Therapeutic dose) i.e, 260 x10 =2600mg/kg body weight as a highest dose through oral route. The animals were observed for the general behavior, ataxic gait, nystagmus, cyanosis, spontaneous convulsions, pinna reflex, death etc.[18,19]

Evaluation of Hepatoprotective activity of (SAJ)

The method of drug induced model was followed. 24 male Wistar rats were divided into four groups. The hepatotoxicity in these animals was induced by Paracetamol.[20,21,22,23,24,25,26] The rats were divided into four groups each consist of six rats.[14,15,18,19,27]

The control group was given only sterile normal saline. The toxicant group was treated with paracetamol (3gm/kg) for the first day to produce toxicity in the liver. The standard group (Liv-52 treated) was given a single dose of paracetamol (3gm/kg) on the first day and then Liv-52 (500mg/kg/daily) was given for 7 days. The test group received a single dose of paracetamol (3gm/kg) on the first day followed by oral treatment with a daily dose (180mg/kg) of SAJ for seven days.

Group – I	Control group	Sterile normal saline
Group – II	Toxicant group	Paracetamol (3gm/kg, single dose on day 1 in normal saline suspension)
Group- III	Standard group	Paracetamol (3gm/kg single dose on day 1) + Liv52 (500mg/kg for 7 days in sterile normal saline suspension)
Group- IV	Test Group	Paracetamol (3gm/kg single dose on day 1) +SAJ Treated (180 mg/kg for 7 days) in sterile normal saline suspension.

Sample collection

The blood was collected on 0 day and after 7 days of the experiment from orbital sinus under anesthesia. The following biochemical parameters were assessed by using standard methods.[28,29,30]

1.Haemoglobin(Hb).

2.Serum Glutamic Oxaloacetic Transaminase (SGOT)/Asparatate Amino Transferase.(AST)

3.Serum Glutamic oyruvate transamiase (SGPT)/ Alanine Amino Transferase (ALT).

4. Serum Bilirubin (SB).

5.Alkaline phosphate (ALP).

Histopathological evaluation[31]

The animals were sacrificed by cervical dislocation under anesthesia with chloroform and the liver was collected and rinsed in normal saline to remove the blood from its surface. The liver samples were fixed in 10% buffered neutral formulation. Thin sections of the formalin fixed liver embedded in paraffin of 5-6 microns were made. They were stained with hematoxylin-eosin (H&E) staining and observed under microscope (100X).

Statistical analysis

The mean values and standard deviation were calculated for each parameter. For determining the significant inter group differences each parameter was analyzed separately. ANOVA was performed to test whether there is any significance in the test group and the rest of the groups. DUNNET ONE side test was employed to check the statistical significance for the test group vs rest of the groups. As parameters were non –gaussian, Mann-Whitey test (Non- parametric test) was performed to obtain significant difference, if any between the groups.

RESULT

SAJ a poly herbomineral formula was found to possess excellent *in vitro* antioxidant activity as represented in Tables 2 & 3 and Graph 1. Acute oral toxicity study revealed that SAJ is a safe compound as none of the animals died during the observation period and there were no sign and symptoms of toxicity related to it as represented by Table 4 and its maximum tolerance dose (MTD) more than 2600mg/kg in mice indicating its high safety profile. SAJ at a dose of 180 mg/kg was found to have significant ($p<0.05$) hepatoprotective activity by the inhibition of elevated biomarkers of paracetamol induced liver damage *viz,* SGOT, SGPT, ALP and SB apart from maintaining normal hemoglobin levels and was found to be comparable with that of Liv 52 which was confirmed by the histopathological examination of

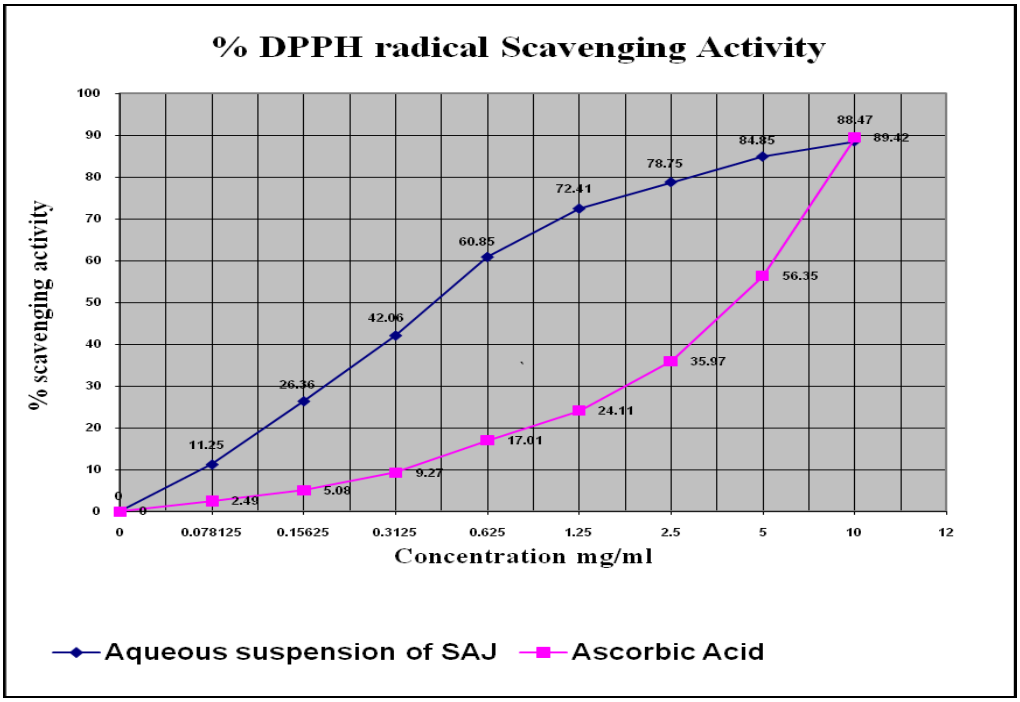
liver of rats and also revealed no pathological changes even after administration of SAJ for 7 days.

The result after various statistical tests was found to be encouraging and the data indicates that SAJ at the dose of 180mg/kg has significantly ($p<0.05$) expressed the anti-hepatotoxic effect against paracetamol induced liver damage by restoring the elevated serum marker enzymes as represented by Table 5 & Graph 2, apart from maintaining the normal haemoglobin level as represented by Graph 3.

Table 2: Antioxidant activity of SAJ (DPPH radical scavening activity)

Conc.(mg/ml In DMSO)	%Inhibition of SAJ± Standard Deviation	%Inhibition of Ascorbic Acid (standard) Mean±SD
0	0	0
0.7825	11.25±2.13	2.49±1.91
0.15625	26.36 ±1.56	5.08±2.11
0.3125	42.06±1.38	9.27±1.79
0.625	60.85±0.65	17.01±0.62
1.25	72.41±1.20	24.11±1.99
2.5	78.75±0.57	35.97±2.04
5	84.85±0.26	56.35±1.29
10	88.47±0.25	89.42±2.48

(SC 50 value of Antioxidant activity of SAJ *Vs* Ascorbic acid)



Graph 1: Antioxidant activity of SAJ (DPPH radical scavenging activity)

(SC 50 value of Antioxidant activity of SAJ *Vs* Ascorbic acid)

Table 3: Test species/doses/duration.

Test details	Test species	Dosage Regimen		Study duration
		Dosage level	frequency	
Acute toxicity	Mice	10 x Therapeutic dose (260)	Once	14 days
Hepatoprotective activity	Rats	10 x Therapeutic dose (180 mg/kg body wt.)	Once a day for 7 days	7 days.

Table 4: Toxicological Signs and Symptoms observed in experimental animals.

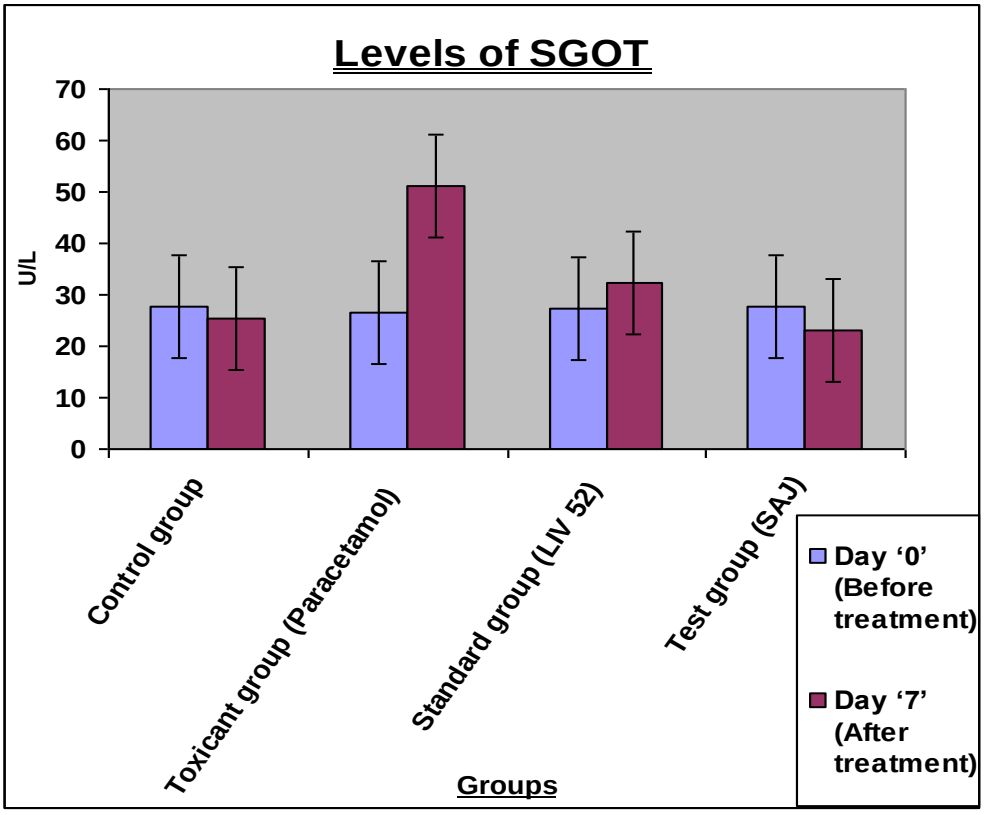
Observation	MICE (2600 mg/kg)									
	Males					Females				
Mice No.	1	2	3	4	5	1	2	3	4	5
Bizarre physical position	X	X	X	X	X	X	X	X	X	X
Bizarre tail position	X	X	X	X	X	X	X	X	X	X
Muscle tremors	X	X	X	X	X	X	X	X	X	X
Aggressiveness	X	X	X	X	X	X	X	X	X	X
Inactivity	X	X	X	X	X	X	X	X	X	X
Spontaneous convulsions	X	X	X	X	X	X	X	X	X	X
Dyspnea	X	X	X	X	X	X	X	X	X	X
Sedation	X	X	X	X	X	X	X	X	X	X
Nystagmus	X	X	X	X	X	X	X	X	X	X
Abnormal Gait	X	X	X	X	X	X	X	X	X	X
Abnormal Excreta	X	X	X	X	X	X	X	X	X	X
Abnormal Salivation	X	X	X	X	X	X	X	X	X	X
Nasal Discharge	X	X	X	X	X	X	X	X	X	X
Piloeretion	X	X	X	X	X	X	X	X	X	X
Altered Muscle Tone	X	X	X	X	X	X	X	X	X	X
Catatonia	X	X	X	X	X	X	X	X	X	X
Change in Pupil size	X	X	X	X	X	X	X	X	X	X
Altered sensitivity to Pain	X	X	X	X	X	X	X	X	X	X
Skin lesions	X	X	X	X	X	X	X	X	X	X
Corneal opacity	X	X	X	X	X	X	X	X	X	X
Righting reflexes	X	X	X	X	X	X	X	X	X	X
Ataxic gait	X	X	X	X	X	X	X	X	X	X
Cyanosis	X	X	X	X	X	X	X	X	X	X
Lacrimation	X	X	X	X	X	X	X	X	X	X
Paralysis	X	X	X	X	X	X	X	X	X	X
Death	X	X	X	X	X	X	X	X	X	X

√ **-Positive, X – Negative**

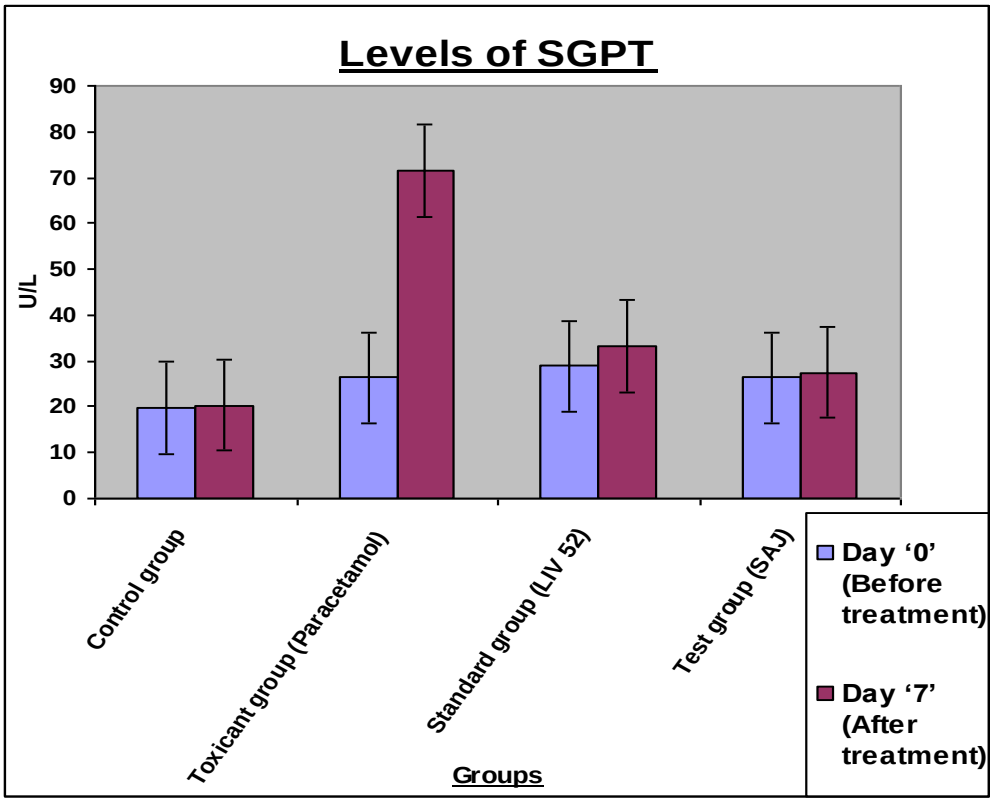
Table 5: Hematological and biochemical parameters before and after SAJ treatment (Mean).

Group	Day '0' *(Before treatment)*					Day '7' *(After treatment)*				
	Hb gm%	**SGOT U/L**	**SGPT U/L**	**S.B g/dl**	**ALP U/L**	**Hb gm%**	**SGOT U/L**	**SGPT U/L**	**S.B g/dl**	**ALP U/L**
Control group	10.76 ±1.9	27.83± 9.7	19.67± 6.4	0.71± 0.4	98.83 ±12.0	10.81± 1.8	25.50± 4.5	20.33± 7.2	0.47± 0.1	105.1±66.5
Toxicant group	11.48 ±1.96	26.50± 7.14	26.34± 7.14	0.47± 0.2	89.34 ±24.3	11.45± 1.92	51.16± 17.1	71.50± 25.9	1.08±0.4	166.84± 22.74
Standard group (LIV52)	11.71 ±1.63	27.16± 9.95	28.84± 10.0	0.45± 0.2	145.17±62	11.60± 1.48	32.17± 15.0	33.17± 9.88	0.56±0.9	175.84± 52.31
Test group (SAJ)	11.30 ±1.41	27.84± 9.98	26.34± 12.5	0.63± 0.5	118.5 ±55.2	11.28± 1.38	23.17± 8.52	27.50± 11.7	0.55±0.1	122.17± 22.25

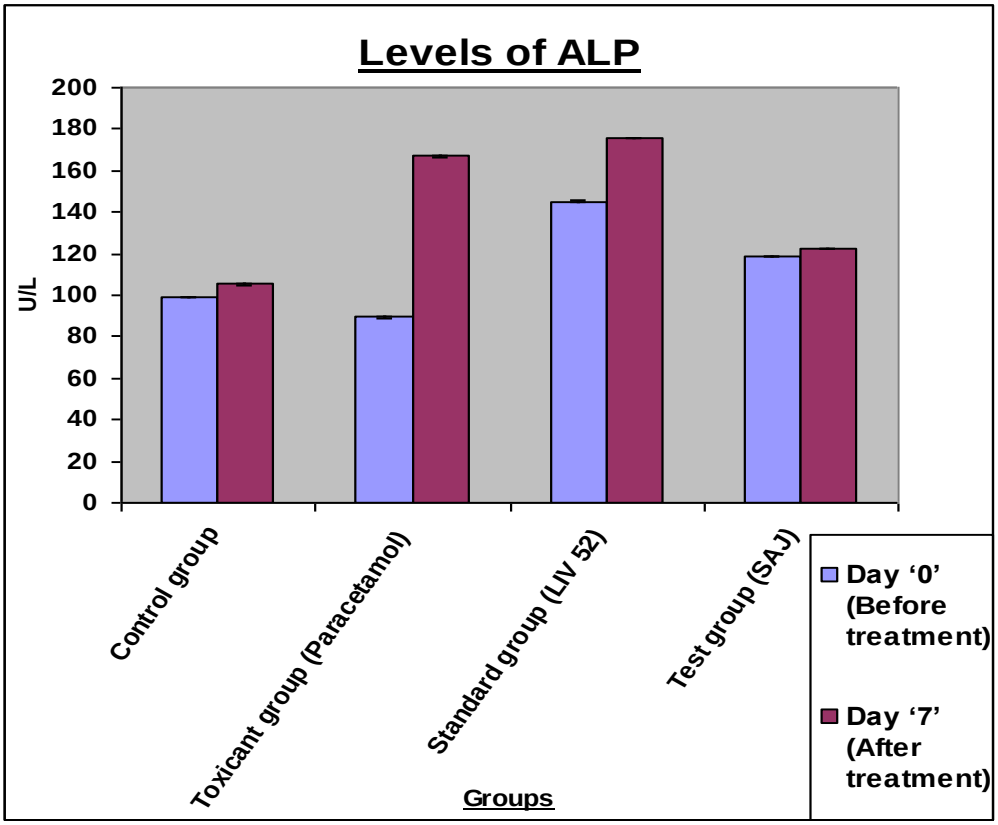
Hb: Haemoglobin; **SGOT**: Serum Glutamic Oxaloacetic Transaminase; **SGPT**-Serum Glutamic Pyruvate Transaminase; **SB**-Serum Bilirubin **ALP** - Alkaline phosphatase.



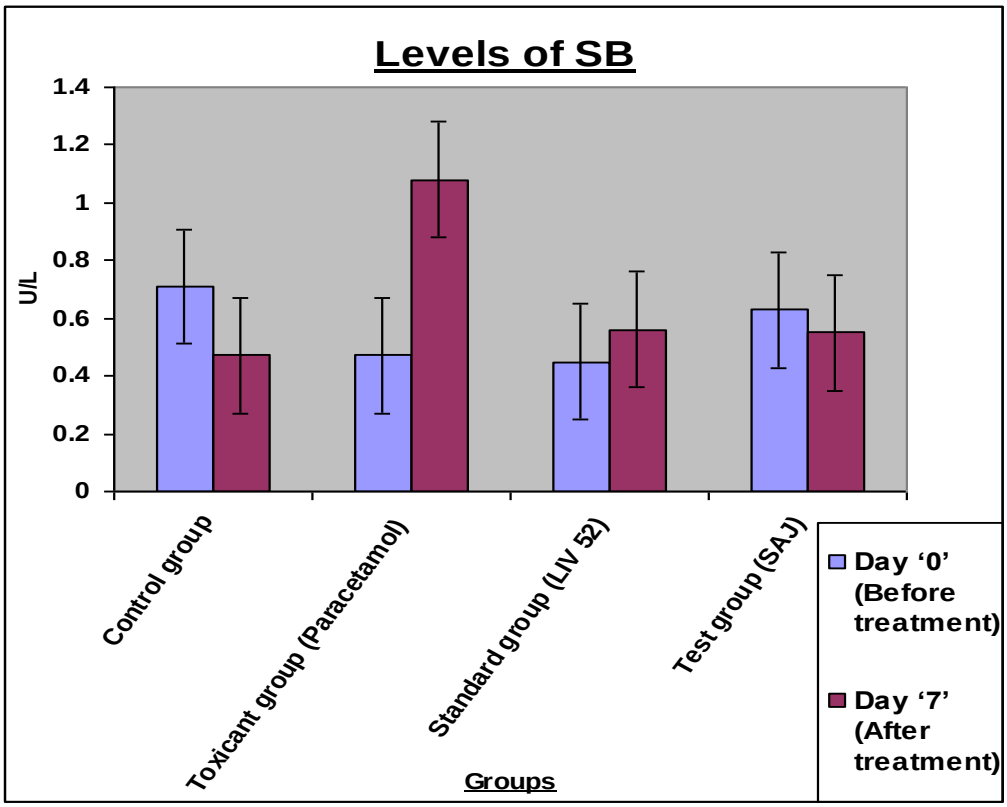
(i)


Levels of SGPT
U/L
90
80
70
60
50
40
30
20
10
0
Control group
Toxicant group (Paracetamol)
Standard group (LIV 52)
Test group (SAJ)
Groups
Day '0' (Before treatment)
Day '7' (After treatment)

(ii)

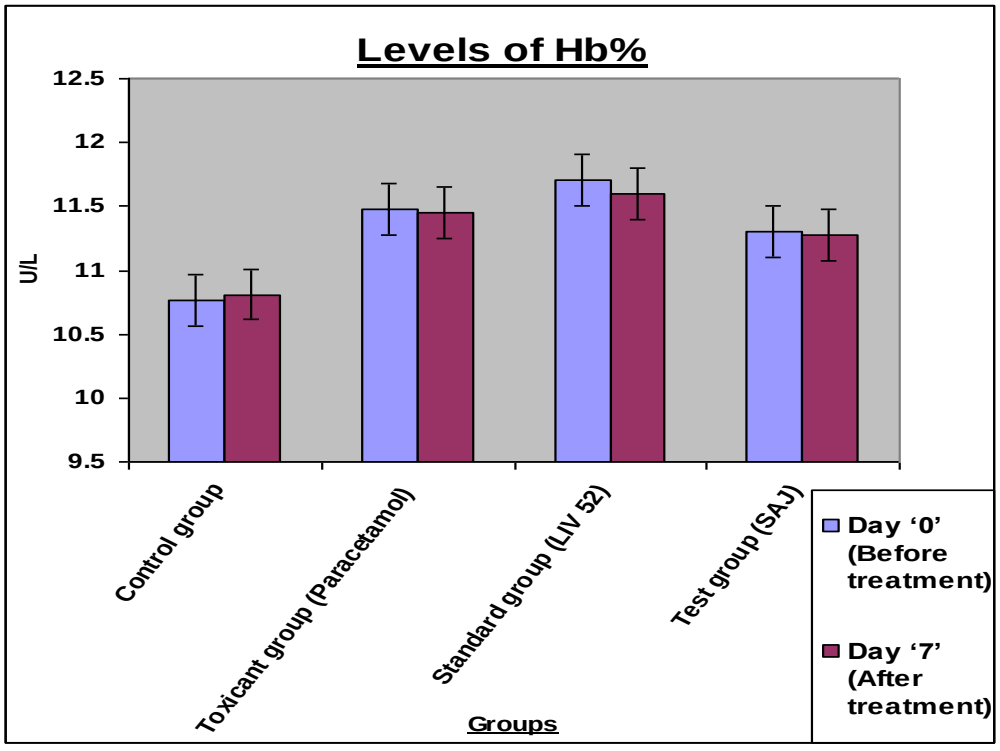

Levels of ALP
U/L
200
180
160
140
120
100
80
60
40
20
0
Control group
Toxicant group (Paracetamol)
Standard group (LIV 52)
Test group (SAJ)
Groups
Day '0' (Before treatment)
Day '7' (After treatment)

(iii)



(iv)

Graph 2 (i-iv): The levels of various biochemical parameters before and after treatment with SAJ.



Graph 3: The levels of hemoglobulin before and after treatment of SAJ.

Histopathology

Changes such as diffuse necrosis and degeneration of hepatocytes, focal edema of the liver in control, toxicant, standard and test groups were observed as shown in the Figure 1. Control group showed normal architecture of hepatic parenchyma with normal hepatocytes. Toxicant (Paracetamol treated) group had the hepatotoxicity in the form of focal areas of vaculation and degeneration of few hepatocytes as evident from the parameters studied. Standard (Paracetamol and Liv 52 treated) group rats showed normal architecture of hepatic parenchyma with normal hepatocytes indicating the hepatoprotective effect of Liv 52 [31]. SAJ also showed normal architecture of hepatic parenchyma with normal hepatocytes indicating a hepatoprotective effect of the test compound and has brought back to normal liver and it has antagonized the hepatotoxicity induced by the paracetamol. Paracetamol-induced liver necrosis was inhibited and restored significantly by SAJ, which confirms the protective action of the SAJ against experimentally induced liver damage in rat.[7]

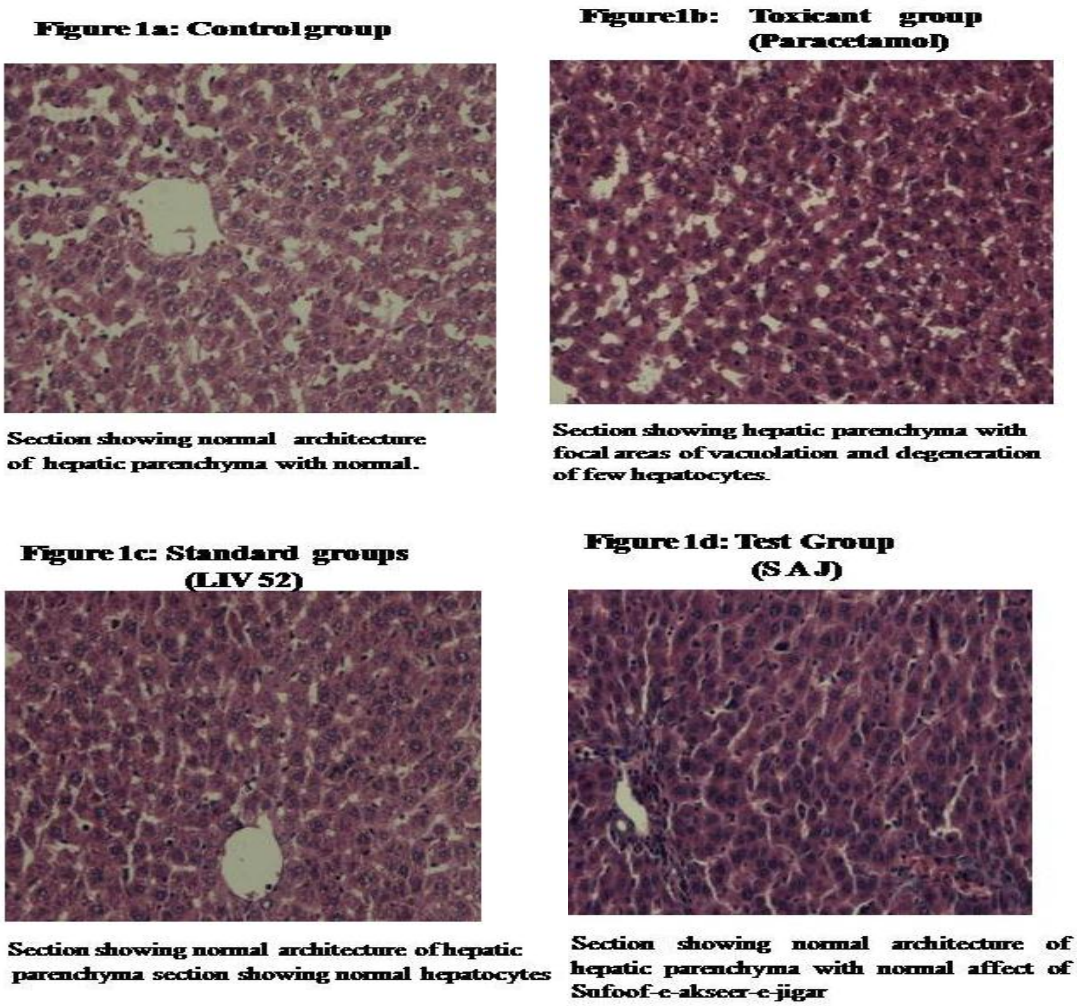


Figure 1a: Control group
Section showing normal architecture of hepatic parenchyma with normal.
Figure 1b: Toxicant group (Paracetamol)
Section showing hepatic parenchyma with focal areas of vacuolation and degeneration of few hepatocytes.
Figure 1c: Standard groups (LIV 52)
Section showing normal architecture of hepatic parenchyma section showing normal hepatocytes
Figure 1d: Test Group (S A J)
Section showing normal architecture of hepatic parenchyma with normal affect of Sufoof-e-akseer-e-jigar

Figure 1: Changes such as diffuse necrosis and degeneration of hepatocytes, focal edema of the liver in control, toxicant, standard and test groups.

DISCUSSION

In Unani medical stream, a great variety of plants are used for medicinal treatments. Having a thorough evaluation of the history of medicine it can be stated that, when this ancient Unani system of medicine is surviving since so long, it means that it proves to be the fittest system of medicine to survive in the present competitive worlds. It was the ancient Unani physicians whose efforts offered the modern world to think over and workout the rest of the needful.

Even, the world is looking for an alternative medicine to cooperate with the present challenges for the health of society. As the world is fed up with the toxicities and side effects of modern medicine and the world is seriously looking for the alternative medicine which need to be efficient, economical and nontoxic to maintain health.

Even though, the therapeutic value of herbal drugs, especially Unani drugs, is established in view of clinical experience of hundreds of years, the scientific assessment of the extent of potency of a drug and the mode of action could however fortify the claims made. Moreover, herbal medicinal products may vary in composition and properties. Correct identification and quality assurance of the starting material is, therefore, an essential prerequisite to ensure the safety and efficacy of the herbal drugs. Scientifically validated and technologically standardized herbal medicines may be derived using a safe path of reverse pharmacology approach based on traditional knowledge database. This may play a vital role in drug discovery, development and therapeutics, in addition to dealing with a typical Western bias against herbal medicine.

Free radicals are chemical species of atoms or molecules that possess an unpaired electron on their outmost orbital. These free radicals are highly unstable and can therefore react with other molecules by giving out or accepting single electron. The human body; although continuously produces free radicals, it also possesses several defense systems, which are constituted to enzymes and radicals scavengers. Although oxidation reactions are critical for life, they can also be damaging; hence, plants and animals maintain complex systems of multiple types of antioxidants, such as glutathione, vitamin C, and vitamin E as well as enzymes such as catalase, superoxide dismutase, and various peroxidases. Too low levels of antioxidants or inhibition of the antioxidants enzymes cause oxidative stress and may damage or kill the cells.[32]

Most of the antioxidant potentials in herbs and spices are due to the redox properties of phenolic compounds that allow them to act as reducing agents, hydrogen donators and free radicals quenchers.

Non-viral hepatitis is an inflammation of the liver due to drug toxicity. Acetaminophen (paracetamol) is one of the drugs most commonly used 'over the counter' analgesic for headache, musculoskeletal pain, dysmenorrhea etc. On the other hand, if taken in larger doses it can also produce serious toxicity especially to liver (9, 10). There is an ever-increasing need of an agent, which could protect it from such damage. In view of severe undesirable side effects of synthetic agents, there is growing focus to follow systemic research methodology and to evaluate scientific basis for the traditional herbal medicines that are claimed to possess hepatoprotective activity. Moreover, it may also be predicted that a good anti-oxidant drug can be a precursor of Hepatoprotection.

The present work would help to promote the awareness towards this ever-reliable ancient system of Unani medicine that it contains wonderful drugs, which act sufficiently without any side effect. The finely grinded powder of Sufoof-e-Akseer-e-Jigar (SAJ), which is a compound of Revand (Rheum emodi), Naushadar (Ammonium chloride) and Shora (Potassium nitrate) is used to evaluate In-vitro, its Anti-oxidant potential; and In-vivo, its Acute toxicity and safety profile in experimental mice; and also to evaluate In-vivo, its Hepatoprotective activity in Paracetamol induced liver damage in experimental rats.

In the present study, the trial drug i.e, Sufoof-e-Akseer-e-Jigar (SAJ) was able to reduce the stable radical DPPH to the yellow-coloured diphenylpicryl hydrazine. Further, the calculated SC_{50} values also showed encouraging results. There were no toxic symptoms even at the highest dose of 2600 mg/kg of SAJ. Hence, the maximum tolerated dose was found to be >2600 mg/kg. Moreover, the LD_{50} value by the oral route could not be determined as no mortality was observed until the dose of 10 gm/kg of SAJ declaring it as very safe.

The collected data indicates that 'SAJ' has significantly expressed the anti-hepatotoxic effect of SAJ against paracetamol induced liver damage by restoring the elevated serum marker enzymes.

Histopathological studies were carried out on the liver samples. Paracetamol-induced hepatocytes degeneration was inhibited and restored significantly by SAJ, which confirms the protective action of the SAJ against experimentally induced liver damage in rats (Figure 1).

At this stage, however, we do not know the exact mechanism responsible for hepatoprotective effects, but considering the individual drug's efficacy in various subjects (animals and humans) like, (a) efficacy of Rheum as a viricidal[33], and its excellent results in acute and chronic Hepatitis, Hepatic coma, Hepatic cirrhosis, etc[34,35] (b) efficacy of Ammonium chloride in inhibiting infection of Viroprex penetrating viruses, Inflenza A & B viruses[36,37], an its very good response as an anti-tumor agent[38], etc, and (c) efficacy of Potassium Nitrate in cirrhosis, liver inflammations and hepatomegaly[39,40,41] and their combined activities in the current study, we are able to designate it as an effective Anti-oxidant and Hepatoprotective agent. Further studies using some more models of experimental hepatic damage are required to elucidate exact molecular and biochemical mechanisms involved in its role as a Hepatoprotective agent.

CONCLUSION

The present studies demonstrate the Safoof-e-Akseer-e-Jigar (SAJ) a poly herbo-mineral formula was found to possess excellent antioxidant activity. It was also found to be safe when administered internally through oral route in animal model. The histopathological studies confirm that the SAJ at a dose of 180 mg /kg was found to have significant ($p<0.05$) hepatoprotective effect against paracetamol induced their damage.

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